

PET hydrolysing enzymes catalyse bioplastics precursor synthesis under aqueous conditions

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Poly(ethylene terephthalate) hydrolase (PETase) was used to catalyse a double transesterification to convert FDCA dimethyl ester to the bioplastics precursor bis-butandiol ester in good yield under aqueous conditions. The presence of high water content was important for high transesterification selectivity. CAL-A and CAL-B lipases showed low activity or extensive hydrolysis. The PETase bioconversion was demonstrated at gram scale and the transesterification products can be selectively extracted for use in polymerisation reactions.

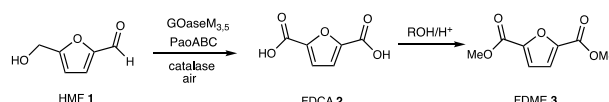
Plastics are essential materials and are found in almost every aspect of everyday life. However, most plastics are non-renewable and not biodegradable and therefore present major challenges in terms of environmental pollution. There is an urgent need to develop both sustainable manufacturing and recycling strategies using bio-renewable feedstocks. If current production and waste trends continue, roughly 12,000 Mt of plastic waste will be in landfill or in the natural environment by 2050.¹ PET is a widely used petrochemically derived plastic composed of terephthalate (TPA) and ethylene glycol (EG). Bio-derived alternatives such as poly(ethylene furanoate) (PEF),² developed by Avantium and now Synvina in a joint venture with BASF,³ are on the market although the current total bio-based market is currently still only around 4Mt.¹ Thus there is a great deal of scope for development and growth of bio-renewable, biodegradable plastics.

PEF and related bioplastics are made from cellulose-derived furan-2,5-dicarboxylic (FDCA) esters by reaction with aliphatic diols. The polymerisation reaction is done using titanium or antimony metal catalysts under high vacuum at elevated temperatures. The reactions are often done in two stages:

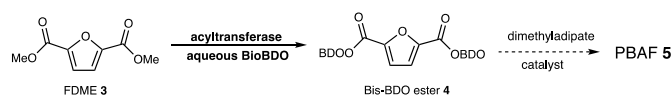
initially a lower temperature (ca 200°C) reaction gives small oligomeric esters, then a high vacuum and higher temperature is applied in a second stage to fully form the high MWT polymer.^{4,5} PBAT (EcoFlex), a biodegradable polymer made by BASF, is a copolyester of butan-1,4-diol, adipic acid and terephthalic acid. It is manufactured by block polymerisation of the two parent polyesters (adipate-BDO and terephthalate-BDO). PBAF, poly (butylene adipate-cobutylene 2,5-furandicarboxylate), is the furanoate analogue and is fully bio-derived and biodegradable since furanoate, unlike terephthalate, is bio-derived.^{5,6} A two-stage enzymatic approach using *Candida antarctica* lipase B has been used to polymerise dimethyl furanoate with aliphatic diols in the high boiling solvent diphenyl ether,⁷ although the final molecular weights are lower than the chemical process, most likely resulting from the limited lifetime of the enzyme at the required elevated temperatures of ca 90°C.

We have recently shown that the cellulose-derived HMF **1** can be oxidised to polymer precursor FDCA **2** in high yield and efficiency using two oxidase enzymes galactose oxidase M_{3,5} (GOaseM_{3,5}) and periplasmic aldehyde oxidase (PaoABC) (Scheme 1).^{8,9}

Previous work



Current work



Scheme 1 Proposed route to bio-renewable plastic PBAF.

FDCA can be readily converted in high yield to the dimethyl ester FDME **3** using a Fischer ester synthesis and DuPont and ADM have recently announced a new process for conversion of fructose directly to **3**.¹⁰ For the synthesis of bio-based copolyesters such as PBAF **5**, pre-formation of the bis-butandiol FDCA ester (bis-BDO) **4** using an enzyme reaction would be highly advantageous. We envisaged that this could be achieved using a suitable acyltransferase enzyme to catalyse a

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transesterification, under aqueous conditions, allowing use of bio-based BDO¹¹ without removal of water, following its production by fermentation. In the normal co-polymerisation to make PBAF, simple adipate diesters such as dimethyladipate are more reactive with 1,4-butanediol (BDO) than the simple furanoate (methyl or ethyl) esters, leading to irregular random polymers. This usually results in a decreased melting point, degree of crystallinity and tensile strength.⁵ Thus, pre-formation of the more reactive bis-BDO ester **4** to replace slower reacting simple FDCA esters would give a faster and more controlled polymerisation and more uniform co-polymer. The search for enzymes that can achieve high acyl transferase activity using low cost esters under significantly aqueous conditions is an important challenge.^{12–14} Lipase enzymes such as *Candida antarctica* lipase B (CAL-B), are known to catalyse ester bond formation, usually in hydrophobic organic solvents under low water conditions. However, in general the use of hydrolases for ester bond formation in higher water conditions is scarce. It has been noted that some lipases such as CalAc5 and CpLIP2 give kinetically higher rates of acyl transferase activity (transesterification) *versus* hydrolysis for long chain fatty acids under semi-aqueous conditions and that the initially formed ester products then subsequently hydrolyse to give a thermodynamic mixture of hydrolysis and acyl transferase products.¹⁵ A similar kinetic preference for transesterification with ethyl acetate or vinyl acetate has been noted using the acyltransferase from *M. smegmatis* (MSAcT) in water.^{16,17} Differences in active site conformation of oxyanion-loop residues of lipases/esterases *versus* acyl transferases have been proposed to account for the differences in activity.^{18,19}

In 2016, the enzyme PETase was isolated from the bacterial strain *Ideonella sakaiensis*²⁰ which can grow on microcrystalline PET as a sole carbon source. PETase can hydrolyse PET under mild conditions (30°C) in aqueous buffer to give monohydroxyethyl terephthalate (MHET), terephthalic acid (TA) and ethylene glycol (EG). Other hydrolases such as cutinases²¹ from *Thermomyces insolens* (HiC),²² *Thermobifida fusca* (TfCut1 and TfCut2),^{23–25} LC cutinase, *T. fusca* hydrolases TfH²⁶ and TfH BTA-2²⁷ and lipases such as CAL-B are also known to degrade PET but with lower activity than PETase.

In this paper we compared the use of PETase and TfH hydrolases and lipases CAL-A and CAL-B for the transesterification of FDCA dimethyl ester (FDME) **3** with butanediol under aqueous conditions. We show, for the first time, that PETase and TfH can give unexpectedly high transesterification selectivity to give mono and bis-butandiol (BDO) esters rather than hydrolysis products in 50:50; buffer:BDO and that the efficiency of the transesterification reaction actually relies on having a high water content in the solvent. In contrast, the widely employed CAL-A and CAL-B lipases give either very low conversion or significant hydrolysis under these conditions.

Transesterification *versus* Hydrolysis for PETase, TfH, CAL-A and CAL-B

It is known that many hydrolases can catalyse transesterification by acyl transfer in organic solvents, usually hydrophobic solvents containing only sufficient water to retain the active conformation of the enzyme. The ratio of water to other nucleophile (BDO in this case) in the reaction mixture can influence the selectivity. However, acyltransferase activity in water miscible alcohol mixtures is usually compromised and high water activity would normally favour hydrolysis. We investigated PETase from *Ideonella sakaiensis* and TfH from *Thermobifida fusca*, based on their known ability to catalyse the hydrolysis of aliphatic-aromatic polymers.^{20,26} These enzymes have never been reported to catalyse ester formation under any conditions. As a comparison we used two lipases, CAL-A and CAL-B from *Candida antarctica*.²⁸ These lipases are known to catalyse both hydrolysis and transesterification of esters, depending on the solvent/water content. The activity and selectivity for transesterification *versus* hydrolysis of the four enzymes (TfH, PETase, CAL-A and CAL-B) was compared using FDME **3** as the substrate, initially in 1:1 buffer:BDO over 24h (Figure 1). Direct monotransesterification would give mixed ester **6** followed by a second exchange to give the desired Bis-BDO ester **4**. Alternatively, hydrolysis reactions would give the undesired half methyl ester **7** and monoBDO **8**.

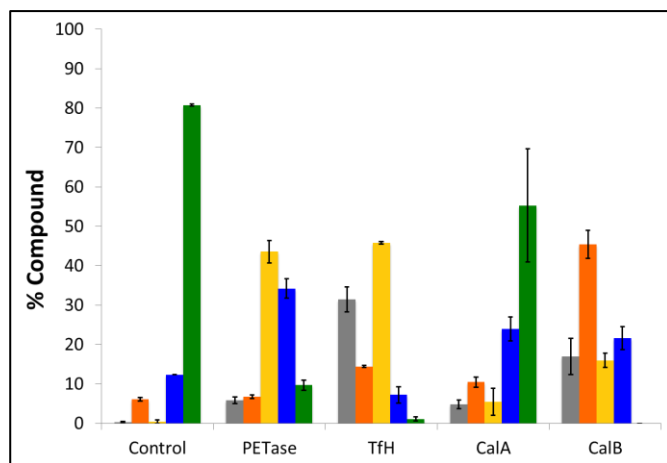
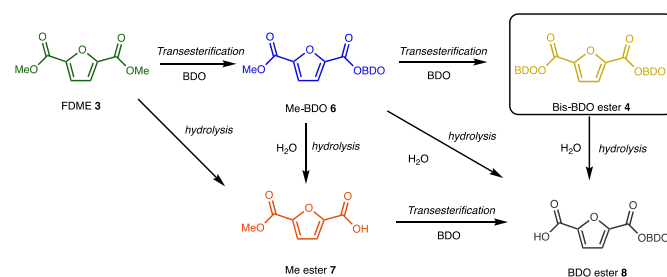


Figure 1. Bioconversion FDME **3** (10mM) with enzyme (1μM) in Tris-HCl buffer (50mM)/BDO (1:1) at 25°C after 24h. Product ratios as percentage of total: Orange - monomethyl ester **7**; grey - mono BDO ester **8**; yellow - Bis-BDO ester **4**; blue - Methyl BDO ester **6**; green FDME **3**.

In addition to high conversion to Bis-BDO ester **4**, high total transesterification to mixtures of **4** and the mono-transesterified product **6** would also give useful precursor mixtures for downstream polymerisation.

PETase showed the highest overall acyltransferase activity, with a total amount of 77% transesterified products (**6** + **4**), with *ca* 45% of Bis-BDO **4** (Figure 1). For TfH, a similar amount of **4** was formed, but less **6** (7%) with more significant amounts of monoesters **7** and **8** resulting from unwanted hydrolysis. CAL-A gave low activity with virtually no Bis-BDO **4** while CAL-B showed higher activity although with a preference for hydrolysis products, with 45% of monoester **7** and 17% of monoBDO ester **8**. Since the acyl transferase/hydrolase activity can be significantly altered by changing the reaction conditions, we determined the effects of pH and temperature for both hydrolases and lipases at the 1:1 solvent ratio over 24 h (ESI Figure S2). Using PETase, best results for high transesterification selectivity for formation of **6** + **4** were observed at pH 8-8.5, with no appreciable bioconversion at pH7 (the range tested was 7 - 8.5, see ESI), whereas pH higher than 9 triggered a high level of chemical hydrolysis. 25°C was the optimum temperature, with higher reaction temperatures (37 or 42°C) suppressing the Bis-BDO ester formation and the only significant product at 37°C was that resulting from monotransesterification to give **6** (ESI Figure S2). The unwanted mono esters (BDO mono ester **8** and Me monoester **7**) resulting from hydrolysis were below 10% at all temperatures and pHs.

The conversion with TfH showed lower selectivity for transesterification and a very different kind of temperature and pH dependence (ESI Figure S3). Modest transesterification selectivity was seen at pH 7.5 with increasing hydrolysis at higher pH, especially at 37°C where MonoBDO ester **8** formed in 70%. The temperature dependence difference between PETase and TfH prompted us to carry out CD thermal analysis on PETase (ESI Figure S8). The *T_m* of PETase was determined to be 40±0.34°C; this is consistent with the very low activity of PETase noted at 42°C. The *T_m* of *T. fusca* cutinase 2 (86% identity to TfH) is 68 ±0.14°C, thus TfH is able to function at all temperatures tested. Despite the presence of two disulphide bonds in PETase, which have been previously described to increase the stability of polyester hydrolases,²⁹ PETase showed a lower *T_m* compared to the *T. fusca* enzyme which is known to be thermophilic.²⁰

The use of CAL-A gave comparable results (ESI Figure S4) to the no enzyme control (ESI Figure S6), with a very low activity (maximum conversion of FDME **3** ~20% to methyl BDO diester **6**). Conversion of FDME **3** using CAL-B gave a very different outcome with a predominance of the hydrolysis products monomethyl ester **7** and MonoBDO **8** particularly at higher pH (8-8.5), with some Bis-BDO ester (ESI Figure S5). Interestingly, CAL-B conversion was not affected by temperature to the same extent as PETase and TfH. Thus, the widely used CAL-A and B enzymes, known to catalyse transesterification on simple esters under organic or even semi-aqueous conditions, are not effective in the transesterification of these substrates and support the use the 'depolymerase' type enzymes PETase and TfH. The very high degree of selectivity for transesterification over hydrolysis that these enzymes afford in

50% buffer/diol is remarkable and is likely to result from the hydrophobic binding cleft that these enzymes possess.^{23,30-33}

Effect of buffer:BDO ratio on selectivity of PETase

In order to gain further insight into the selectivity we determined the effect of changing the ratio of Buffer:BDO on the product distribution over 24h using PETase (Figure 2). A 1:1 ratio gave the optimum overall (*ca* 75%) transesterification (Bis-BDO ester **4** + Me BDO ester **6**) whilst minimising hydrolysis products. However, notably at higher water content (9:1; buffer:BDO), the predominant product was the MonoBDO ester **8** (*ca* 70%) rather than the monomethyl ester **7** or diacid FDCA **2** (not detected). Therefore **8** is not a good substrate for hydrolysis by PETase. Interestingly this correlates to the published selectivity of PETase that is unable to hydrolyse mono hydroxyethyl terephthalate (MHET).²⁰ Unexpectedly, at higher levels of BDO in the solvent (e.g. 1:4 Buffer:BDO ratio), reaction of the starting substrate **3** is much slower and formation of the Bis-BDO ester **4** is almost precluded. Increasing the amount of BDO in solution beyond 50% appears to inhibit the enzymatic activity and doesn't significantly change the degree of hydrolysis, showing that using a partially aqueous solvent system (above 20% water) is absolutely essential to achieving double transesterification to form Bis-BDO ester **4**.

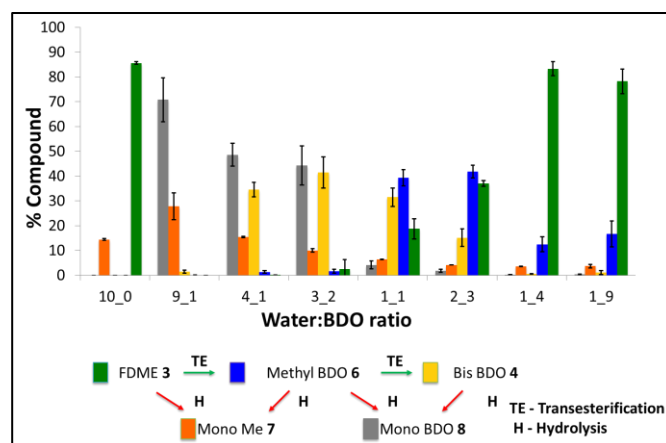


Figure 2. Conversion of **3** using PETase in varying ratios of Buffer:BDO: **3** (10 mM) with PETase (1μM) in Tris-HCl buffer (50 mM)/BDO (ratio) at 25°C. Product ratios as percentage of total after 24h.: Orange - mono ester **7**; grey - mono BDO ester **8**; yellow – Bis-BDO ester **4**; blue - Me BDO ester **6**; green FDME **3**.

Time course reactions, comparing the 4:1, 1:1, 3:7 buffer:BDO (Figures 3 A-C) gave greater detail on the sequence of the reactions involved, along with comparison of overall conversion of transesterification and hydrolysis product (ESI figure S7). FDME **3** reaction with PETase at 1:1 buffer:BDO (Figure 3B) clearly shows a stepwise conversion of FDME **3** *via* methyl BDO ester **6** to the Bis-BDO ester **4**. Monotransesterification of **3** to give methyl BDO ester **6** is favoured over hydrolysis (to give monomethyl ester **7**). This is closely followed by the second transesterification to give Bis-BDO for the active site of PETase, since this

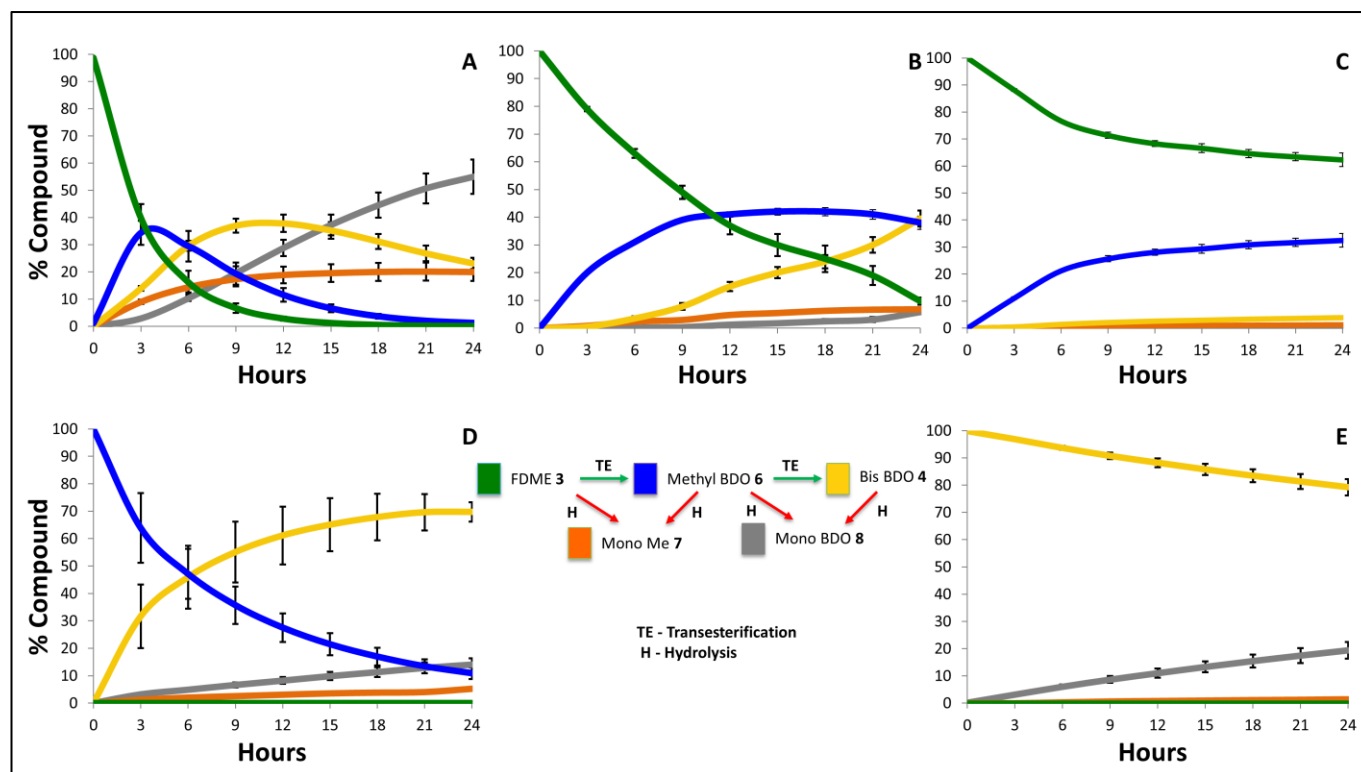


Figure 3. 24 h analysis of FDME **3** (10mM) conversion with 4:1 buffer:BDO (**A**), 1:1 buffer:BDO (**B**), 3:7 buffer:BDO (**C**) with PETase (1 μ M) in Tris-HCl buffer (50 mM)/BDO (ratio) at 25°C. Conversion of (**D**) MeBDO **6**, (**E**) Bis-BDO **4**, (10mM) using PETase (1 μ M) in Tris-HCl (50 mM)/BDO (1:1) over 24h. Product ratios as percentage of total after 24h.: Orange – mono Me ester **7**; grey – mono BDO ester **8**; yellow – Bis-BDO ester **4**; blue – Me BDO ester **6**; green FDME **3**.

second transesterification starts early in the time course (6h). The rate of formation of monomethyl ester **7** is always very slow and is likely to be formed from FDME rather than by hydrolysis of the BDO ester group of methylBDO ester **6**, since its rate of formation does not match methylBDO **6** formation. The time course using 4:1 Buffer:BDO (Figure 3A) shows clearly that the initial rate of transesterification to form methyl BDO ester **6** and Bis-BDO ester **4** is faster than hydrolysis, even with higher water content. However, hydrolysis then occurs to give mono methyl and mono BDO esters **7** and **8**. Increasing the concentration of BDO in the reaction (3:7 Buffer:BDO, Figure 3C) results in a lower rate of conversion to methylBDO **6** followed by a tailing off of activity and very little Bis-BDO **4** formation. Analysis of time courses over a range of water:BDO ratios (see ESI S7) suggests that a water:BDO ratio of 1:1 gives the best overall selectivity for transesterification products without dramatically compromising the enzyme activity.

Substrate selectivity of PETase

The time course results prompted us to run reactions comparing the activity of PETase on the transesterification

intermediates, MeBDO **6** and Bis-BDO **4** (Figure 3D and E) with FDME **3** (Figure 3B) using the 1:1 ratio of Buffer:BDO. The intermediates were synthesised as substrates and standards by chemical transesterification (ESI S9). As observed in Figure 3B, and 3D, the transesterification reaction is always favoured when compared with hydrolysis; both FDME **3** and MeBDO ester **6** are converted to Bis-BDO **4**, whereas hydrolysis is slow. In particular, MeBDO **6** seems not to be converted to monoBDO **8** ester, since **8** is not formed in appreciable amounts before Bis-BDO starts to accumulate. This suggests that **8** is formed exclusively from Bis-BDO **4**. Indeed, Figure 3E shows that Bis-BDO **4** is a substrate for the formation of monoBDO **8**, although the reaction is slow. The formation of monomethyl ester **7** also appears to be very slow (Figure 3D and 3E), suggesting that monoBDO **8** is not transesterified by PETase using methanol. There is also no evidence for transesterification of monomethyl ester **7** to give monoBDO ester **8** even at longer incubations where the concentration of this intermediate remains constant (ca 10%) (see ESI Figure S2B for 7 days incubation). Hence, our results show that for PETase, the transesterification rate is higher than the

hydrolysis rate, confirming the surprising preference for acyl transfer when using 50% aqueous conditions.¹⁵ This would allow isolation of the combined transesterified products (**6** and **4**) at an optimum yield, whilst minimising hydrolysis products. Even approaching the thermodynamic equilibrium, at extended reaction times (7 days, ESI Figure S2B) the Bis-BDO ester content seems to be constant at around 60%, although the unwanted MonoBDO ester **8** reaches ca 20%, likely to result from sequential conversion of residual MeBDO **6** to Bis-BDO **4** to monoBDO ester **8**.

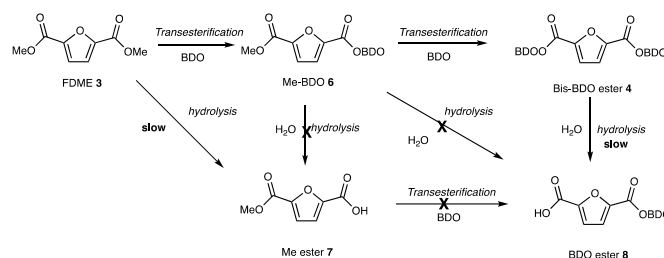
Preparative Scale Reaction

In order to demonstrate the transesterification on a preparative scale, 1g of FDME **3** (5.4 mmol) was biotransformed (see below). At this concentration we found that pH control in the optimal range (8–8.5) was important to enable the reaction to reach completion. The transesterification products (63% total **6** + **4**) were isolated following extraction with ethyl acetate and evaporation to give a transparent oil (1.39g) comprising of Bis-BDO **4** (0.8g, 59%), and a small amount of MeBDO **6** (40mg) in BDO. This crude extract mixture is ideally suited as a starting mixture for the chemo-catalysed polymerisation reaction.

Conclusion

Two enzymes PETase and Tfh, both recently identified as hydrolases for the hydrolysis of aromatic-aliphatic polyesters, show remarkably high acyl transferase activity under 50% aqueous conditions for double transesterification of the simple furan diester FDME **3**. This substrate can be bio-derived from cellulose, thus paving the way for synthesis of bioplastic polyesters from cellulose. Diesters such as the Bis-BDO ester **4** are promising intermediates for chemical-catalysed copolymerisation with aliphatic monomers such as dimethyl adipate, since the Bis-BDO ester is 'pre-armed' as a diol for polymerisation and more reactive than the dimethyl ester. Interestingly, the widely used *Candida antarctica* lipases CAL-A and CAL-B failed to deliver activity and selectivity for the transesterification reactions of aromatic diesters. CAL-B is known to possess a water tunnel, connecting the solvent to the active site, facilitating hydrolysis even when the enzyme active site is facing the lipid phase.³⁴ WT CAL-A and an improved variant showed high acyltransferase activity for palm kernel oil with aqueous ethanol.³⁵ CAL-A has been widely reported to prefer long-chained carboxylic acids and small acid groups.³⁶ The crystal structure of CAL-A revealed the presence of a narrow ~30-Å-long tunnel near the catalytic residues which is closed by an alpha helix³⁷ hindering aromatic substrate access. Related enzymes CalAC5, CduLac, CpLip2 were recently shown to give high transesterification in aqueous methanol compared to CAL-A.¹⁵ However, these enzymes work on aliphatic substrates and share relatively low sequence identity with PETase and Tfh (ESI S10). Therefore, the very low activity observed by CAL-A towards furan diesters

prompted us not to consider other CAL-A related enzymes for processing aromatic diesters. Recent detailed structural and mechanistic studies on PETase have shown that this enzyme has a hydrophobic cleft that is ideally adapted to bind the aromatic-aliphatic polyester substrate chain.³⁰ Mutagenesis experiments and docking of the PET tetramer model substrate 2-HE(MHET)₄ showed that the enzyme contains a catalytic triad (Ser160-His237-Asp206) in subsite I of the active site where Ser160 acts as the nucleophile for formation of the acyl-enzyme intermediate via the enzyme-stabilised oxyanion. Subsites IIa-c accommodate the rest of the chain with key residues providing additional substrate binding. Subsite I residues are highly conserved in related enzymes (e.g. Tfh) although the adjacent subsite IIa has significant differences, giving increased space for accommodation of the second MHET (monohydroxyethyl terephthalate) residue in PETase. From the results presented here, under optimised conditions, we propose as the major pathway that FDME **3** is converted by PETase to MeBDO diester **6**, then to Bis-BDO diester **4** (Scheme 2). FDME **3** or MeBDO **6** undergo very slow or negligible hydrolysis to the monomethyl ester **7**. Bis-BDO **4** ester undergoes slow hydrolysis to monoBDO **8** but only up to ca 20% after 7 days. MeBDO diester **6** does not appear to be hydrolysed to monoBDO **8**.



Scheme 2: Pathway for conversion of FDME **3** catalysed by PETase showing transesterification pathways dominating over hydrolysis.

The PETase enzyme cleft appears to have sufficient space to accommodate all the substrates and intermediates we have analysed in this study. The unexpectedly high acyl transferase selectivity in the presence of 1:1 water:BDO and even higher water levels, may result from preferential binding of BDO over water at the active site. This would also help to explain the apparent stability of Bis-BDO **4** over prolonged reaction times, where it could keep undergoing transesterification with more BDO. This may slow down the expected thermodynamic equilibration to hydrolysis products. Yoshida²⁰ found that PETase was not capable of hydrolysing MHET and this is consistent with our finding that monoBDO **8** is not hydrolysed and fortunately only accumulates slowly. Relatively higher desolvation energy for binding polar monoacid intermediates such as MHET and monoBDO **8** may account for the apparent inactivity on these substrates. Future mutagenesis efforts in our laboratory will aim to eliminate the formation of monoBDO **8** as well as increasing the rate of formation of Bis-BDO **4**.

Compared with TfH and related enzymes, PETase has an additional disulphide bond near the active site (Cys203 and Cys239) and an extended loop ($\beta 8-\alpha 6$) which helps accommodate the polymeric substrate. Both TfH and PETase show a degree of thermostability which is known to correlate with tolerance to organic solvents. This may explain why these enzymes appear to work so well under what would normally be hostile conditions for most lipases which prefer either aqueous, hydrophobic solvents or biphasic conditions rather than water miscible organic solvents. In the transesterification reaction, the relative rates and product profile suggest that process engineering may maximise the benefits of the reaction sequence observed. For example, continuous addition of FDME could increase the rate of production of Bis-BDO by minimising the concentration of FDME **3** which is otherwise in competition with the faster reacting MeBDO ester **6**. Also, continuous removal of Bis-BDO ester **4** may speed up the preceding conversions, again by removing possible product inhibition. The prospect of an entirely bio-based route to biodegradable bioplastic co-polymers such as PBFAF is attractive. Bio-BDO (30,000t/a) is manufactured at Novamont by glucose fermentation.¹¹ After removal of the cells, the crude product is purified prior to removal of water by distillation. Our ability to transesterify FDME to form the Bis-BDO ester selectively under aqueous conditions means that Bio-BDO could be used directly without the removal of water, thus reducing the cost significantly. The Bis-BDO ester **4** may then be selectively extracted using a suitable solvent prior to polymerisation with bio-based dimethyl adipate. We have demonstrated the potential for scale up of this bioconversion and that the transesterification products can be selectively extracted using ethyl acetate. Current work is focussed on increasing the selectivity of extraction using greener solvents and use of the enzymatically synthesised Bis-BDO ester **4** for copolymerisation with dimethyl adipate to make PBFAF.

Representative procedure of FDME **3** conversion to Bis-BDO ester **4**

PETase was expressed in *E. coli* BL21 RIPL strain grown in 1L 2YT media with induction by IPTG. The enzyme was purified by Ni-affinity chromatography and concentrated before use as a solution. FDME (1g, 5.4 mmol, final concentration = 108 mM) and PETase (0.5 mL of 2.2 mg mL⁻¹, final concentration = 7 μ M) were added to 25 mL 1,4 butanediol, 1.21 g Tris, 0.29 g of NaCl and H₂O to a final volume of 50 mL. The reaction was vigorously shaken at 25 °C for 24 hours to allow solubilisation of FDME. The pH was constantly monitored and adjusted using 1M NaOH during the reaction to maintain a pH 8-9 and avoid the pH falling below 7.5. After this time, FDME was converted to 63% of transesterification products. The solution was extracted with ethyl acetate (5 x 50ml) before concentration *in vacuo* yielding an oil comprised of Bis-BDO in BDO/water solution (0.8 g of Bis-BDO and 40 mg MeBDO) based on HPLC using a standard curve (ESI S10).

Enzymatic Assay and Analysis

Activity assay experiments were carried out in 50 mM Tris-HCl buffer, mixing 1 μ M enzyme and 10 mM substrate, at different pH, temperatures and water:BDO ratio. The reactions were quenched by adding 2% TFA and any protein precipitate was removed by centrifugation 5 min at 13,000 x *g*. The mixture was finally diluted 5x and the sample analysed using an Agilent 1260 Infinity II LC System High-performance liquid chromatography (HPLC). Samples were loaded on HyperClone™ 5 μ M ODS (C18) 120 Å (125 x 4.0 mm). All the analyses were performed at room temperature (25°C). The mobile phase was constituted by applying a 30 min gradient from Buffer A (5% methanol + 95% water + 0.1% TFA) to Buffer B (90% methanol + 10% water + 0.1% TFA) at a flow rate of 0.6 mL min⁻¹. The compounds of interest were detected by measuring the absorbance of the furan ring at 254 nm. Quantification and identity of the compounds was determined by HPLC by comparison with chemically synthesized standards FDCA **2**, FDME **3**, MeBDO **6**, monomethyl ester **7**, BDO ester **8** and Bis-BDO **4** (ESI S10).

Conflicts of interest

There are no conflicts of interest to declare.

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